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STRUCTURAL RECOGNITIONS IN THE INTERACTIONS OF ANDROGENS AND RECEPTOR PROTEINS AND IN THEIR ASSOCIATION WITH NUCLEAR ACCEPTOR COMPONENTS

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SUMMARY

5α-Dihydrotestosterone can bind to specific receptor protein(s) in the microsomal or cytosol fractions of rat ventral prostate. The binding is highly steroid specific and correlated with the androgenicity of the steroids. A large part of the androgen molecule appears to be physically enveloped by the receptor protein molecule. Since certain derivatives of 17β-hydroxy-estra-4-ens also bind to the receptor protein, the detailed atomic arrangements and local electronic structure do not seem to be as important as the gross solid-geometric structure of an androgen for its ability to bind to the receptor molecule.

A prior interaction and binding of 5α-dihydrotestosterone to the cytoplasmic receptor protein is required for the retention of the latter by cell nuclei of prostate. The nuclear retention of the cytoplasmic protein-5α-dihydrotestosterone complex is dependent on a heat-labile protein (nuclear acceptor) in the nuclei. The acceptor molecule is a nuclear acidic protein which appears to bind to double-stranded DNA, poly G or poly A and to a much lesser extent to poly U, poly C or heat-denatured DNA. Some possible biological implications of these studies are presented.

INTRODUCTION

IN LINE with the current concept that steroid hormones act by regulating certain gene activities in the cell nuclei of target tissues, various steroid hormones have been found to associate closely with the nuclear chromatin of the responsive cells. Such nuclear retention of a steroid hormone appears to be preceded by an interaction of the hormone with a specific receptor protein in the cell cytoplasm It is believed that the steroid-receptor complex, rather than the steroid alone, is retained by the cell nuclei.

This paper summarizes our work of the last few years on the interactions of androgens and cellular macromolecules in rat ventral prostate. Discussions will be centered on the structural recognition of molecules involved in the interactions. Review articles and papers are available for a more comprehensive treatment of the related subjects and findings of other investigators not referred to in this paper (see Refs. [1, 2] and other papers in this issue).

5α-DIHYDROTESTOSTERONE BINDING PROTEINS

In 1968, it became known that 5α-dihydrotestosterone (DHT)*, a metabolite of testosterone, can be retained by cell nuclei of rat ventral prostate *in vivo* [3–5] or *in vitro* [4, 5] for a prolonged length of time. These observations prompted

*5α-dihydrotestosterone or DHT: 17β-hydroxy-5α-androstan-3-one.

the search for the specific protein(s) which may be qualified as the androge receptor(s). It is now clear that the cytoplasmic soluble fractions as well as crud extracts of nuclei or microsomes from prostate contain proteins which can bin DHT with some degree of specificity and affinity [5–10].

It is not known at this time the precise relationship of these DHT-bindin proteins which may exhibit different sedimentation constants or be retained b various cellular sites under a variety of experimental conditions. Much of th difficulty is due to the fact that during these studies, the androgen-protein comple is identified by following the radioactive DHT and not the function of the protei or the complex. This makes it almost impossible to relate various DHT-bindin proteins described by different investigators or even by the same worker.

β-PROTEIN

In our laboratory, an extensive study has been made on the cytoplasmi DHT-binding protein which can be retained by the prostate cell nuclei [1, 2, 5 11–14]. To distinguish it from other DHT-binding proteins, we have named th protein β-protein [13, 14]. For this study, we have developed a cell-free systen in which one can demonstrate the retention of a cytoplasmic DHT-protei complex by the purified prostate cell nuclei [5, 11–14].

The cell-free system is highly specific for steroid-protein complexes retaine by cell nuclei and also demonstrates the tissue specificity expected from *in viv* experiments. Stoichiometric studies have also shown that such a system can b used to measure qualitatively and quantitatively a specific DHT-binding protei [14].

The β-protein-like protein(s) can be extracted from the cytoplasmic particu lated fractions (including microsomes, see Refs. [1, 2, 11]). In complex wit [^{3}H]DHT, these proteins (in a 0·4 M KCl solution) migrated with sedimentatio constants in the vicinity of $3S_{20,w}$ which is indistinguishable from the [^{3}H]DHT protein complex extracted from the prostate nuclei of rats injected with [^{3}H]DH or [^{3}H]testosterone [1, 5, 6, 14]. These DHT-protein complexes gradually aggre gate in the absence of KCl at 0°C.

STRUCTURAL REQUIREMENTS FOR AN ANDROGEN TO BIND TO β-PROTEIN

β-Protein is highly steroid specific. At low concentrations [< 1 n M], steroid such as 5β-dihydrotestosterone, testosterone, androstenedione, 5α-androstane 3,17-dione, 5α-androstane-3α or 3β, 17β-diol, progesterone, cortisol, or estradiol 17β do not compete well with the binding of [^{3}H-DHT to β-protein [11–14] Thus, β-protein appears to act as a specific selector in deciding which natura steroids can or cannot be retained by cell nuclei in the target tissue. The competi tion study suggests that, on the steroid molecule, C-3 should not have a hydroxy group and C-17 should not have a carbonyl group. Whether oxygen function a either of these positions is required for the binding at all is not clear. It is interest ing to note that androgens lacking C-3 oxygen are known, whereas the 17β hydroxy configuration seems to be essential for androgen activity [1]. Since 5β dihydrotestosterone is not bound to β-protein, it appears that the bound steroi molecule must have a rather flat steroid carbon skeleton. At C-7, methyl substitu tion at the equatorial position [7β] strongly hinders the binding, whereas methyl group at the axial position [7α] usually enhances [see below] the bindin

ability. Some restrictions on the peripheral sides of the steroid molecules are, therefore apparent. Available information indicates that there is a good correlation between the structural requirements for a steroid to bind to β-protein and to show androgen activity in rat prostate.

From the fact that DHT has a much higher affinity than testosterone for β-protein, one may assume that the unsaturated bond in ring A is not desirable for the binding. This, however, is not consistent with our observation that various 19-nor-4-androstene derivatives are equally, or more, effective than DHT in binding to β-protein or showing androgenicity*. For example, competition experiments show that 7α-methyl-19-nortestosterone and 7α,17α-dimethyl-19-nortestosterone bind to β-protein with three to four times the affinity that DHT does [13]. Since the unsaturated bonds at C-4 of these compounds are not reduced under the conditions of the study, we conclude that the inability of testosterone to bind firmly to β-protein is not due to the undesirable electronic structure associated with the unsaturated bond.

One plausible explanation is that the androgen binding site on the β-protein is inside a narrow hole of the receptor with a limited width and can accomodate the flat molecules like DHT, but not testosterone (Fig. 1). If, however, the thickness of the steroid is reduced by the removal of the 19-methyl group, the reduction of the unsaturated bond may not be necessary. The increase in receptor binding affinity due to the substitution of a 7α-methyl group on 19-nortestosterone is in accord with the increase in the androgenicity due to the same substitution [15]. It is possible that a specific binding site (see Fig. 2) normally not involved in the binding of DHT is present in the β-protein for the binding of the 7α-methyl group and thus both the binding affinity and the androgenicity is enhanced many times.

It is interesting to note that the terminal methyl group on the diethylstilbestrol may behave like the 7α-methyl group on the 19-nortestosterone in enhancing the binding affinity to the estrogen receptor and the estrogenicity. This may also imply a similarity in the androgen receptor and estrogen receptor in their hormone binding sites and their importance in biological action.

NATURE OF DHT BINDING TO β-PROTEIN

Several workers have previously attempted to use a semiempirical approach by comparing chemical structure and end-point activity, to predict the way androgens might interact with hypothetical receptors (see reviews in Ref. [1]). Suggestions were made for the binding of androgens by the receptors from α-face, β-face and/or peripheral attachments. The information obtained by these workers was obviously complicated by the structural requirements for various selection processes involving various macromolecular bindings.

The structural considerations described above and other experiments with our β-protein preparation strongly support the concept that a large part of the steroid molecule is physically enveloped in the receptor protein molecule [2, 13, 14]. In addition, it is very likely that the detailed atomic arrangements and local structures are not as important as the gross solid-geometric structure of an androgen for its ability to bind to β-protein. To illustrate this point, we made a plastic box having nine flat faces, just enough to accommodate a CPK molecular

*This work was carried out in collaboration with Dr. J. C. Babcock of The Upjohn Company, Michigan, U.S.A.

model of DHT, but not testosterone (Fig. 2). Two holes were made to accommodate the 7α and 17α methyl groups of 7α, 17α-dimethyl-19-nortestosterone. Other steroids described above and not able to bind to β-protein do not fit the geometry of the box. Interestingly, potent androgens, such as A-nor-17β-acetoxy-4,9,11-estratrien-3-one, 2-oxa-17β-acetoxy-4,9,11-estratrien-3-one, B-homo- and D-homo-5α-dihydrotestosterone can be accommodated in the box, but weak or non-androgenic steroids such as 5β-dihydrotestosterone or A-homo-5α-dihydro testosterone do not fit in the box. The nine-faced box may thus reflect a maximum geometry allowed for an androgen to bind to β-protein and exert androgenic action through such binding.

If the steroid molecule is physically enveloped in the receptor protein molecule (presumably in part by certain polypeptide chains with limited flexibility), a change in the protein conformation may be expected during the binding of DHT. Such a change may, in fact, be the primary effect of DHT *in vivo*, and as a result, the complex will have the structural requirement to fit the functional site, for example, the 'nuclear acceptor' site. Supporting evidence for this has been presented in our previous reports in which β-protein retention by isolated prostate nuclei was shown to be enhanced by a prior interaction and binding of DHT to β-protein. A part of the steroid molecule may also recognize the 'acceptor' sites, but it is also possible that the steroid molecule is not directly involved in the interactions (with the 'acceptor' materials or with the other nuclear components) needed for the induction of the biological response [2, 14].*

"NUCLEAR ACCEPTOR" SUBSTANCES

The retention of the DHT-receptor complex by the prostate nuclei is highly specific toward steroids, proteins, and tissues. Since the ability of the nuclei to retain DHT-receptor ('acceptor' activity) is minimized by treatment with a proteolytic enzyme or by heat treatment, we have suggested that the nuclei of the target tissues may contain a protein factor or factors which determine the nuclear sites where the steroid-receptor is retained. The factor(s) is called 'nuclear acceptor(s)' [11–14].

We have found that when cell nuclei of the target tissues are extracted with a 0·4 M KCl solution, the extract contains not only the steroid-receptor complex, but also proteins which appear to show 'nuclear acceptor'-like activity. For the assay of the 'acceptor' activity, nuclear extract obtained from the castrated animals (the extract, therefore, contains very little receptor) is incubated with a [^{3}H]DHT-receptor preparation. The precipitates are collected by centrifugation (with a proper amount of histone or heated liver nuclear extract as a carrier) or by a filtration through a millipore membrane [no carrier needed]. The precipitates are washed and then extracted with 0·4 M KCl to dissociate the [^{3}H]DHT-receptor complex, and the radioactivity is measured [16].

The prostate nuclear 'acceptor' activity as defined by our assay procedure, is associated with the protein fractions which are not dialyzable. Like the 'acceptor' activity of whole nuclei, the 'acceptor' activity of the soluble extract is inactivated by heating for 10 min at a temperature above 50°C or by treatment with trypsin or pronase. Further study is needed to determine how many nuclear components are required for the specificity and 'acceptor' activity. Equivalent liver nuclear extracts appear to contain lesser amounts of the nuclear acceptor substance(s).

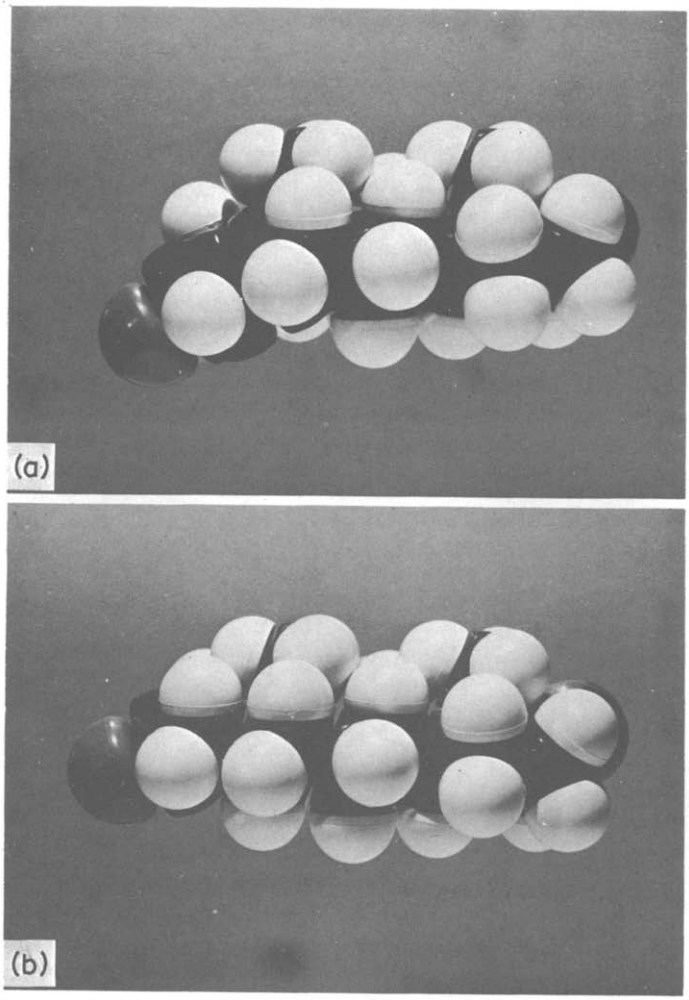

Fig. 1. CPK molecular models (Ealing Corporation) of testosterone (A) and 5α-dihydrotestosterone (B).

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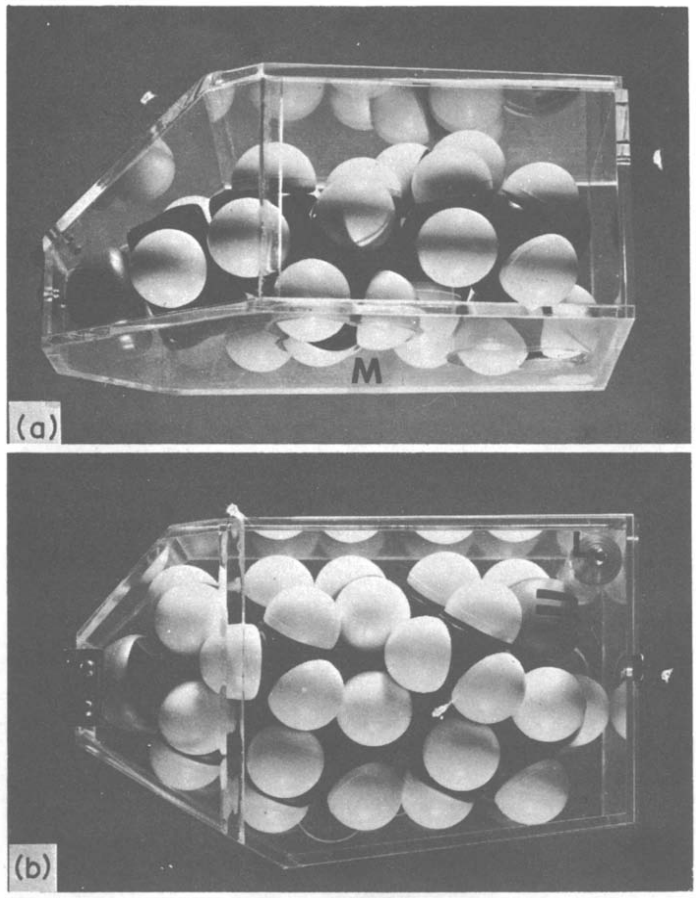

Fig. 2. CPK molecular models of 7α, 17α-dimethyl-19-nor-testosterone (A) and D-homo-5α-dihydrotestosterone in a plastic box (with a hinged cover) made to fit 5α-dihydrotestosterone, but not testosterone. In A, the site for the 7α-methyl group is shown (M). In B, upper right corner, a battery chamber with a light bulb is shown (L). The light goes on when the box cover is closed completely.

By our assay system, the 'acceptor' activity of the nuclear extract can be stimulated 5- to 10-fold by the addition of a proper amount of purified DNA (rat, calf, or *E. coli*). Such a DNA effect, however, is abolished if DNA is denatured by heating. Excess DNA is inhibitory apparently due to the non-specific binding of the DHT-receptor complex directly to DNA. The stimulatory effect of DNA can be mimicked by liver ribosomal RNA, poly G or poly A. Poly U and poly C are far less effective. It is not known whether the 'nuclear acceptor' activity is dependent on certain helical structures of these polymers or indeed on the specific base (purines) components [16].

BIOLOGICAL IMPLICATIONS

Under our experimental conditions an average of about 2 ~ 3,000 molecules of [^{3}H]DHT-receptor complex are retained by one prostate nucleus [13, 14]. Since not all nuclei are equally labeled with [^{3}H]DHT, the number of the available nuclear acceptor sites in some of the responsive cell nuclei may be considerably higher and could be as high as 5,000–10,000 per nucleus.

After castration, the nuclei appear to lose DHT-receptor complex, suggesting that the 'receptor-acceptor interaction' is a dynamic phenomenon. Since, at body temperature, DHT-receptor complex is apparently less stable than the 'nuclear acceptor' and its associated factor(s), it is possible that the continuous cellular function of an androgen may be very much dependent on the ability of the target cells to replenish the DHT-receptor complex to the nuclei. On the other hand, if the androgen-receptor complex is over-supplied, the number of the available 'nuclear acceptor' sites may determine the limit of the androgen action in a particular cell nucleus.

There is no clear-cut evidence that the processes of androgen retention by prostate cell nuclei are involved in the biological function of androgens in the target tissue. In fact, whether such processes also exist in other androgen-sensitive tissues have yet to be investigated. Nevertheless, a good correlation exists between the androgenicity and the structural recognition of various androgenic steroids (and their precursors) by β-protein, strongly suggesting that these processes play important roles in the regulation of certain nuclear functions in the androgen-sensitive tissue. The tissue specificity of the processes and the inhibition of these processes *in vivo* and *in vitro* by anti-androgens [1, 2, 5, 6, 14] also support such a contention.

A biochemical mechanism proposed earlier for the action of steroid hormones [17] is that steroid hormones bind directly with repressor molecules and inactivate the repressors. If this is the case, the need for the steroid-receptor to re-enter the cell nuclei is puzzling. One of the modified mechanisms which can be proposed is that the steroid-receptor complex (rather than the steroid alone) is a de-repressor molecule which inactivates the 'repressor' in the cell nucleus. Alternatively, the steroid-receptor complex may alter or unwind specific super-coiled regions of nucleohistone complex to make the particular sections of genome metabolically active. Other possible mechanisms include the one in which the steroid acts through a 'positive control element' and regulates genetic activity by activating the reserved RNA polymerase or by influencing the molecules involved in the specific association of RNA polymerase to certain sections of the genome [1, 18, 19].

In the models described above, the hypothetical 'nuclear acceptor' may be considered to be one of the structural constituents of the chromatin, a factor working in conjunction with the steroid-receptor, or a repressive element in the gene transcription. The possibility that DNA and RNA recognize distinct sites on the acceptor-DHT-receptor ternary complex is interesting since this may imply a mechanism for the protective removal of RNA from the gene surface.

All of the above hypotheses must be regarded as highly speculative. It must also be considered that the various biological functions of a steroid hormone in a cell may arise from the existence of a multiple trigger mechanism rather than from a single one.

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DISCUSSION

Munck: I know I was looking at the part of the slide you didn't want us to look at, but you had cyclic AMP there. What did you mean by that?

Liao: We believe that prostate cell nucleus makes cyclic AMP which may be involved in certain nuclear functions (*Biochim. biophys. Acta.* **230** (1971) 535).

Munck: Does it influence androgen activity at all?

Liao: There is no doubt that cyclic AMP influences the growth and function of the target cells stimulated by androgens. However, this does not mean that cyclic AMP can substitute for the action of androgens. Singhal *et al.* reported (*Science* **168** (1970) 261) that cyclic AMP produced testosterone-like induction of certain enzyme activities in castrated and immature rats. However, Rosenfield and O'

Malley (*Science* **168** (1970) 253) were not able to stimulate adenyl cyclase activity of rat ventral prostate *in vivo* or *in vitro* with androgens.

Jensen: As I understand it, you have two dihydrotestosterone-receptor complexes in the cytosol: complex II, which is highly specific and will interact with the nucleus to give the nuclear complex, and complex I, which is of less specificity and will not bind in the nucleus to give an extractable nuclear complex. What I find very interesting and unexpected is that, in the presence of complex I, complex II does not bind to the nucleus, even though I itself doesn't bind and presumably can not compete for the binding site. I wondered if you had any explanation of this rather curious phenomenon.

Liao: We really do not know the answer. Complex I could be the precursor of Complex II, or, on the other hand, the degraded Complex II. There may be a large amount of α-protein which competes with β-protein for radioactive 5α-dihydrotestosterone or even for nuclear acceptor sites. I would like to add that in our Complex II preparation, there is more than one form of protein which binds 5α-dihydrotestosterone firmly and in a highly specific manner. Some of them do not seem to be retained firmly by cell nuclei.

Wira: I was wondering if you've had a chance to talk with Dr. O'Malley, to determine whether your two types of cytosol receptor are the same or different from the A and B forms which he finds with progesterone.

Liao: I think he's found that only one of the progesterone receptors binds to DNA.

Wira: Yes, I think he's found that the A form will bind directly to DNA while the B form requires the acidic protein. Do you think you're dealing with the same thing?

Liao: It might be.

Kellie: I noticed in the diagram relating to the β complex, that the β complex collected the dihydrotestosterone, entered the nuclear compartment, performed its function and then came out of the nuclear compartment. As I understand Dr. Jensen's ideas, on the estrogen receptor is that the cytoplasmic estrogen receptor is consumed in the process whereby the estrogen is carried into the nucleus. I wonder, Dr. Liao, whether there was any significance in the way in which you drew this model, representing the β-complex coming out of the nuclear compartment again?

Liao: The dotted lines in my diagram are the ones which have not been proved experimentally. We do not know how the β-protein comes out from nucleus but we know that after castration the amount of β-protein inside the nucleus decreases with the loss of 5α-dihydrotestosterone. The amount of β-protein in the tissue does not decrease as fast as the nuclear β-protein. Therefore, we assume that the loss of the binding protein from cell nucleus is not merely due to the destruction but rather represents a dynamic picture of interaction among steroid, binding protein, and the function sites in the nucleus.

Jensen: Let me comment briefly on this word "consumed." We used this term back in a 1968 paper when we believed, but could not prove definitely, that the nuclear complex actually involved the same protein as the cytosol complex. All we knew was that it was necessary to have the cytosol protein present in order to get the nuclear one and that, in the process of the interaction, the cytosol protein disappeared from the cytosol. Now we know more about things; we know that when we say "consumed", it doesn't mean that the cytosol receptor is destroyed, but that it leaves the cytosol and goes into the nucleus.

Kellie: The reason I posed this question, was that I thought that according to your ideas, the concentration of cytosol receptors ought to decrease as the nuclear receptor appears.

Jensen: Exactly, it does so both *in vivo* and *in vitro*.

Kellie: We have endeavored to check up on this point by measuring the concentration of oestradiol cytosol receptor, but as far as we were able to determine, it doesn't seem to disappear as one would expect during the transfer of the estradiol into the nucleus.

Jensen: A physiologic dose of estradiol in the immature or castrate rats will cause a marked drop in cytosol receptor during the first 4 h after hormone injection; this has been confirmed by Gorski. Apparently this receptor utilization stimulates resynthesis, so after 4 h the level is gradually restored and by 16 h it is back to normal; in fact, it may overshoot a little.